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REDUCTIVE DECHLORINATION OF HIGH CONCENTRATIONS OF 1,2-DICHLOROETHANE, 1,2-DICHLOROPROPANE, AND 1,1,2-TRICHLOROETHANE BY *DEHALOGENIMONAS* SP.

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Civil Engineering

in

The Department of Civil Engineering

by
Andrew Maness
B.S., Louisiana State University, 2010
May, 2012

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ABSTRACT

The research goals presented here were to evaluate the solvent concentrations over which *Dehalogenimonas lykanthroporepellens* strain BL-DC-9^T and *Dehalogenimonas* sp. IP3-3 are able to reductively dechlorinate 1,2-dichloroethane (1,2-DCA), 1,2-dichloropropane (1,2-DCP), and 1,1,2-trichloroethane (1,1,2-TCA). Both of these novel bacteria were isolated from contaminated groundwater at the PetroProcessors of Louisiana, Inc. Superfund site located near Baton Rouge. Both cluster in the phylum *Chloroflexi*, related to, but distinct from, the widely studied, reductively dechlorinating "Dehalococcoides" strains .

Although previous research demonstrated that strains BL-DC-9^T and IP3-3 are able to reductively dechlorinate a variety of polyhalogenated alkanes, the solvent concentration at which the strains are able to dehalogenate the solvents has not been rigorously evaluated until now. The ability of strains to dechlorinate solvents at high concentrations has important implications for remediation of areas where chlorinated solvents remain in the subsurface as dense, non-aqueous phase liquids (DNAPL's). The concentration range over which strains can reductively dehalogenate chlorinated solvents also has important implications with respect to approaches suitable for growing inocula for use in bioaugmentation.

In the research described here, track study experiments and concentration range experiments were setup simultaneously for each species and compound. The track study involved weekly sampling of replicate bottles spiked with an initial contaminant concentration of 2 mM, a concentration previously known to be degraded by these organisms. The concentration range study was set up to take place over the course of 8 weeks, with sampling at t=0 and after 8 weeks of incubation with replicate bottles containing a wide range of initial contaminant concentrations ranging from 0.5 to 15 mM. All experiments were conducted at 30°C.

Both species were shown to dechlorinate 1,2-DCA, 1,2-DCP, and 1,1,2-TCA at initial concentrations at least as high as 8.7 mM, 4.0 mM, and 3.5 mM, respectively. When compared to the concentrations of these contaminants in the area from which these bacteria were isolated, the organisms were shown to be capable of dehalogenating concentrations at least as high as those present. These results have important implications for the remediation of the PPI Superfund site and many other locations around the world.

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

This chapter presents a brief literature review regarding previous studies testing elevated concentrations of chlorinated alkanes followed by an outline of the thesis.

1.1 Introduction

In industry, polychlorinated ethanes and propanes are used as solvents, degreasing agents, and paint removers. They are also globally produced on a massive scale as intermediates during production of other industrially important chemicals (De Wildeman and Verstraete, 2003; Field and Sierra-Alvarez, 2004). Due to spills and inappropriate past disposal methods, these chlorinated organic compounds are prevalent groundwater and soil contaminants. For example, 1,2-dichloroethane (1,2-DCA) is present in at least 570 current or former Superfund sites (ATSDR, 2001), and 1,2-dichloropropane (1,2-DCP) is present in more than 100 Superfund sites (Fletcher *et al.*, 2009). The prevalence of these polychlorinated alkanes as environmental contaminants is of concern because of their known or suspected toxicity and/or carcinogenicity (ATSDR, 2001).

Anaerobic reductive dechlorination, a process in which microorganisms utilize chlorinated organics as electron acceptors, represents a potentially viable method for cleanup of many contaminated sites (Fennell *et al.*, 2001; Major *et al.*, 2002; Christ *et al.*, 2005). Previous studies on reductive dechlorination of halogenated alkanes have generally been conducted in a relatively narrow range of low (e.g., 0.1 to 0.5 mM) contaminant concentrations (De Wildeman *et al.*, 2003; Grostern and Edwards, 2006a, 2006b, 2009; Grostern *et al.*, 2009; Fletcher *et al.*, 2009; Yan *et al.*, 2009a). Contaminant concentrations considerably higher than this range are present at some sites, however, particularly in areas where pollutants remain in the subsurface as dense non-aqueous-phase liquids (DNAPLs) (Bowman *et al.*, 2006; Marzorati *et al.*, 2007; Yan *et al.*, 2009b).

1.2 Literature Review

At the present time, there has been little research into the effects of high concentrations of chlorinated alkanes on pure cultures with the ability to degrade these compounds. There is a much larger body of work associated with the effect of higher concentrations of chlorinated ethenes on microbial bioremediation. (Grostern and Edwards, 2006b)

Earlier research into degradation of higher concentrations of chlorinated alkanes by an enrichment culture containing "Dehalobacter" showed microbial mediated reductive dechlorination occurring at a concentration of 2 mM of 1,2-DCA (Grostern and Edwards, 2009), as shown in Table 3.1. 1,1,1-TCA at a concentration of 1.5 mM was incompletely dechlorinated and a 1,1,1-TCA concentration of 2.2 mM caused reductive dehalogenation to completely stop (Grostern and Edwards, 2006b).

While research into the effects of high concentrations of chlorinated ethanes such as 1,2-DCA and 1,1,2-TCA is sparse, research into the effects of high concentrations of chlorinated propanes is virtually non-existent. Löffler *et al.* (1997) studied the ability of a mixed culture to dehalogenate varying concentrations of 1,2-DCP. These values ranged from 0.4-30 µmol/bottle in a 24 mL bottle. The highest concentration, 30 µmol/bottle, is equal to approximately 3 mM in that configuration. Using their mixed culture obtained from river sediment, they found that this culture demonstrated incomplete dechlorination beginning around 2.1 µmol/bottle, with complete cessation of reductive dechlorination at approximately 9 µmol/bottle.

Among the limited number of microbes known to anaerobically reductively dehalogenate polychlorinated ethanes and propanes are strains of *Dehalogenimonas lykanthroporepellens* (Moe *et al.*, 2009; Yan *et al.*, 2009) and *Dehalogenimonas sp.* IP3-3 (K.S. Bowman, personal communication). These species cluster in the phylum *Chloroflexi*, related to, but distinct from, reductively dechlorinating "Dehalococcoides" strains (Moe *et al.*, 2009). Strains of both

Dehalogenimonas species reductively dehalogenate 1,2-DCP and 1,2-DCA via dichloroelimination reactions with H_2 as an electron donor, forming final products of propene and ethene, respectively (Yan *et al.*, 2009; K.S. Bowman, personal communication).

Table 3.1. Previous Relevant Research and Literature on Reductive Degradation of Chlorinated Ethanes

Author(s)	Year	Compound	Type of Culture	Concentration(s)
Stucki *et al.*	1983	1,2-DCA	enrichment culture	5 mM
Van Den Wijngaard *et al.*	1992	1,2-DCA	*Ancylobacter aquaticus*	1 mM
Herbst and Wiesmann	1996	1,2-DCA	*Xanthobacter autorotophicus*	0-10 mM
deBest *et al.*	1997	1,1,1-TCA	digested sludge	1.30-10 µM
Klecka *et al.*	1998	1,2-DCA	microcosms	0.2-1 mM
Hage and Hartmans	1999	1,2-DCA	*Pseudomonas*	1 mM, 2.5 mM, 5 mM
DeWildeman *et al.*	2003	1,2-DCA	"Desulfitobacterium dichloroeliminanas" DCA1	0.4 mM
Grostern and Edwards	2006a	1,2-DCA and 1,1,2-TCA	enriched culture ("Dehalococcoides")	0.08-0.30 mM
Grostern and Edwards	2006b	1,1,1-TCA and 1,1-DCA	enrichment cultures (*Dehalobacter* and "Dehalococcoides")	0.03-0.30 mM
Duhamel and Edwards	2007	1,1,2-TCE and 1,2-DCA	mixed microbial culture	DCA: 0.70 mM, TCE: 0.25 mM
Grostern and Edwards	2009	1,2-DCA	enrichment culture containing *Dehalobacter* and *Acetobacterium*	0.50 mM, 1 mM, 2 mM
Kocamemi *et al.*	2009	1,2-DCA	biofilm reactor/mixed culture	0.15-0.70 mM
Grostern *et al.*	2009	1,1,1-TCA and 1,1-DCA	enrichment culture (*Dehalobacter*)	0.15-0.54 mM
Yan *et al.*	2009	1,2-DCA and 1,1,2 TCA	*Dehalogenimonas lykanthroporepellens* and "Dehalococcoides"	0.5mM

Although reports of pure culture's abilities to dechlorinate high concentrations of chlorinated alkanes are generally lacking in the literature, Marzorati *et al.* (2007) reported an

enrichment culture referred to as 6VS (originating from groundwater in Italy where there was 1,2-DCA contamination for more than 30 years) that repeatedly dechlorinated 8 mM 1,2-DCA. Also, Grostern and Edwards (2009) detailed an enrichment culture, including a *Dehalobacter* sp. and an *Acetobacterium* sp.*,* capable of dechlorinating 2 mM 1,2-DCA. Though not previously evaluated for chlorinated ethanes or propanes, previous research on chlorinated ethenes has shown that microbial populations reductively dechlorinating chlorinated aliphatic alkenes, particularly perchloroethene (PCE) and trichloroethene (TCE) can maintain their activity and increase contaminant dissolution rates (Cope and Hughes, 2001; Yang and McCarty, 2002; Dennis *et al.*, 2003; Sleep *et al.*, 2006)

Previously literature accounts of *Dehalogenimonas* strains were conducted only at initial chlorinated solvent concentrations of 0.5 mM (Yan *et al.*, 2009). Research reported here was aimed at evaluating the solvent concentration range over which *D. lykanthroporepellens* and *D.* strain IP3-3 can reductively dechlorinate 1,2-DCA, 1,2-DCP, and 1,1,2-TCA.

While studies regarding the ability of pure or mixed cultures to reductively dehalogenate chlorinate alkanes are relatively rare, as described in the preceding pages, there have been some previous studies that investigated the ability of non-reductively dechlorinating organisms to grow in the presence of chlorinated solvents. For example, Bowman *et al*. (2009, 2010) studied the ability of 18 phylogenetically diverse strains from the genus *Clostridium* and found that all of the strains were able to produce hydrogen in the presence of 7.4 mM 1,2-DCA and 2.4 mM 1,1,2-TCA. Some of the strains were able to produce hydrogen even in media containing concentrations as high as 29.7 mM 1,2-DCA and 9.8 mM 1,1,2-TCA. Rao *et al*. (2012) reported that for strains of the Gram positive bacterium *Actinomyces naturae,* concentrations up to 24.4 mM 1,2-DCA and 7 mM 1,1,2-TCA did not have a major adverse effect on growth, while at

higher concentrations, the extent of growth was inhibited. Bae *et al.* (2006) reported that the bacterium *Propionicicella superfundia* was able to carry out fermentation in the presence of 1,2-DCA and 1,1,2-TCA at concentrations to at least 9.8 and 5.9 mM, respectively. Likewise, strains of *Brooklawnia superfundia* were found to carry out fermentation in media containing 1,2-DCA and 1,1,2-TCA at concentrations at least as high as 9.8 mM (Bae *et al.*, 2006). Thus, it is clear that some bacteria have the ability to grow in the presence of elevated concentrations of chlorinated alkanes.

1.3 Thesis Organization

Chapter 2 of this thesis describes materials and methods employed in the laboratory studies. Chapter 3 contains results and conclusions from these studies. Chapter 4 contains overall conclusions and recommendations for future research. Chapter 5 contains a listing of references citied throughout the thesis.

CHAPTER 2 MATERIALS AND METHODS

This chapter provides an overview of the experimental methods and designs used in the research conducted for and discussed in this thesis.

2.1 Chemicals

1,2 Dichloroethane (>99.8% purity), 1,2 Dichloropropane (99%), 1,1,2-Trichloroethane (96%), ethene ($\geq$99.5%), propene ($\geq$99%), and vinyl chloride (>99.5%) were all purchased from Sigma Aldrich (St. Louis, MO).

2.2 Medium Preparation

Anaerobic basal media was prepared as described by Sung, et. al (2003). The anaerobic medium contained the following constituents (per liter): NaCl, 1.0 g; $MgCl_2 \cdot 6H_20$, 0.4 g; $CaCl_2 \cdot 6H_2O$, 0.1 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; KCl, 0.5 g; resazurin, 0.001 g; L-cysteine hydrochloride, 0.25 g; sodium acetate 0.41 g (5 mmol); sodium pyruvate , 0.55 g (5 mmol); sodium lactate 60% (m/m) syrup, 0.7 mL (5 mmol); non-chelated trace element solution, 1.0 mL (Kuever *et al.*, 2005); and 1.0 mL selenite-tungstate solution (Alain *et al.*, 2002).

After the medium was autoclaved under an N_2 atmosphere and cooled, the following components were added aseptically from sterile stock solutions; 30 mL of $NaHCO_3$ solution (84 g/L autoclaved under N_2 atmosphere), 10 mL of titanium citrate solution (24 g/L), (0.1 mL vitamin solution (40 mg/L 4-aminobenzoic acid, 10 mg/L D(+)-biotin, 100 mg/L nicotinic acid, 50 mg/L calcium D (+)-pantothenate, 150 mg/L pyridoxine dihydrochloride, filter-sterilized and stored in 10 mM pH 7.1 sodium phosphate buffer at 4°C), 0.1 mL thiamin solution (1 g/L thiamine chloride, filter sterilized and stored in 25 mM pH 3.4 sodium phosphate buffer at 4°C), 0.1 mL vitamin B_{12} solution (500 mg/L, filter sterilized and stored in distilled water at 4°C). The pH of the medium was adjusted to 7.0-7.5 with 2.4 M HCl (filter-sterilized).

2.3 Track Study Experimental Design

Experiments were carried out in 25 mL sample bottles, each containing 10 mL liquid and 15 mL gas headspace. The sample bottles were sealed with butyl rubber stoppers and aluminum crimp caps. They were then supplied with a headspace containing 80% H_2 and 20% N_2. Either neat, filter sterilized 1,2 DCP, 1,2 DCA, or 1,1,2 TCA was added to create an aqueous phase concentration of approximately 2 mM. Bottles were shaken to thoroughly mix and allowed to equilibrate for at least 24 hours prior to inoculation.

One of the strains tested was *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 (=ATCC BAA-1523^T, =JCM 15061^T). The second strain, designated as IP3-3 (=JCM 17062, =NRRL B-59545), was recently isolated from contaminated groundwater at the PPI Superfund Site located near Baton Rouge, LA. Based on nearly completed 16s rRNA gene sequencing, this latter strain falls within the genus *Dehalogenimonas*, but with 16S rRNA gene sequence identity of only 96% with BL-DC-9 (unpublished data). Both strains were grown in the dark at 30° C with 1.5 mM 1,2-DCP as an electron acceptor until greater than 50% of the initial 1,2-DCP was transformed. These strains were then inoculated into their respective bottles at 3% (v/v) and were incubated at 30° C in the dark under static conditions (i.e., without shaking). Triplicate bottles were sacrificed at one week time intervals and were analyzed using gas chromatography, to quantify 1,2 DCP, 1,2 DCA, 1,1,2 TCA and their potential degradation products (e.g., Propene, Vinyl Chloride, 1-Chloropropane, 2-Chloropropane, Ethene, 1-Chloroethane, 1,1-Dichloroethane)

2.4 Chlorinated Compound Concentration Range Experimental Design

Additional serum bottles, prepared in the same manner as described above were amended with chlorinated solvents to reach initial aqueous-phase concentrations ranging from 0.5 mM to 15 mM after equilibration. Following inoculation at 3% (v/v), the bottles were

incubated in the dark at 30°C for eight weeks without mixing. Negative controls were prepared and incubated in the same manner but without bacterial inoculation. At t=0 and after 8 weeks incubation, gas chromatography was used to quantify the extent of reductive dechlorination.

2.5 Analytical Methods

2.5.1 Chlorinated Solvent Gas Chromatography

Chlorinated solvents and degradation product concentrations were measured using an HP model 6890 gas chromatograph (GC) as described by Yan *et al.* (2009). Briefly, the GC was equipped with a flame ionization detector and GS-GasPro capillary column (60 m × 0.32 mm I.D., J&W P/N 113-4362) with helium as carrier gas with a flow of 3.0 mL/min. Gas headspace samples collected in 100 µL gas-tight glass syringes (Hamilton, Baton Rouge, LA) were introduced via direct injection. Aqueous samples were purged with helium in a Tekmar 3000 Purge and Trap with sample introduction to the GC using a Tekmar 2016 concentrator. Separate gas-headspace samples and aqueous-phase samples were analyzed from each sample bottle. The mass of each volatile compound per serum bottle was then calculated as the sum of the liquid and gas phase concentrations multiplied by the volume of the respective phase.

2.5.2 Hydrogen Gas Chromatography

Hydrogen concentrations in the headspace gas were also measured, using an SRI Instruments model 310 gas chromatograph (Torrence, CA) equipped with a thermal conductivity detector and molecular sieve column (Alltech Molesieve 5A 80/100) as described previously (van Ginkel *et al.*, 2001).

2.6 Standard Curve Preparation

Liquid standard solutions were prepared by dissolving known volumes of neat compound in 160 mL of water in sealed serum bottles with no headspace. After the solutions were mixed on stir plates and allowed to equilibrate overnight, 0.5 mL aliquots were loaded into the purge and

trap autosampler and analyzed as described above. Serial dilutions of ethene and propene were made by injecting known volumes into sealed 160 mL serum bottles. The diluted gas standards were measured via direct injection. Calibration curves were prepared by plotting peak area versus known concentration.

CHAPTER 3 RESULTS AND DISCUSSION

This chapter presents the results from the studies of the two strains, *Dehalogenimonas lykanthroporepellens* BL-DC-9 and *Dehalogenimonas* strain IP3-3, each with 1,2-DCA, 1,2-DCP, and 1,1,2-TCA.

3.1 1,2-DCA Dechlorination as a Function of Solvent Concentration

The quantity of the dechlorination product ethene, determined at the end of the eight week incubation period as a function of initial aqueous-phase 1,2-DCA is shown in Figure 1. For serum bottles supplemented with 1,2-DCA at initial concentrations less than 3.16±0.05 mM and 1.48±0.03 mM (mean ± standard deviation), dechlorination in bottles inoculated with strains IP3-3 and BL-DC-9^T, respectively, was complete with no 1,2-DCA detected at the end of the eight week incubation period. At higher initial 1,2-DCA concentrations (denoted by arrows in Fig. 1), 1,2-DCA remained at the end of eight weeks.

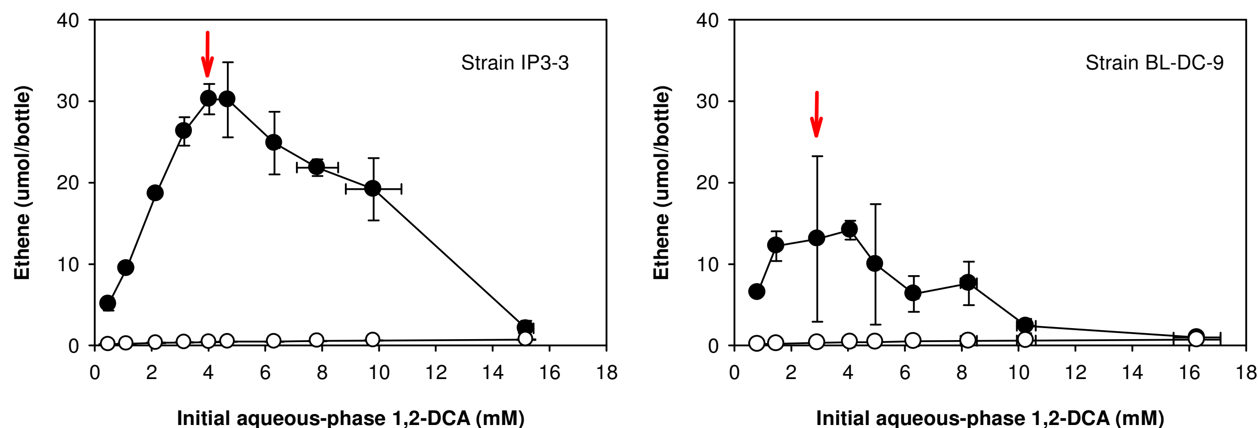

Figure 1. Concentration range study of reductive dechlorination of 1,2-DCA by strains IP3-3 and BL-DC-9^T after 8 weeks. Vertical error bars represent one standard deviation in final measurements of triplicate bottles. Horizontal error bars represent standard deviation of initial concentration in triplicate bottles. Filled symbols denote inoculated (biotic) bottles and open symbols denote uninoculated (abiotic) negative controls.

Trace levels of 1-chloroethane (<0.3 µmol/bottle) were detected in bottles inoculated with strains BL-DC-9^T and IP3-3 and in uninoculated abiotic controls. This indicates that a

small amount of abiotic degradation of 1,2-DCA occurred; however, the amount was negligible. The production of ethene coupled with 1,2-DCA disappearance is consistent with the degradation pathway reported previously for strains BL-DC-9^T and IP3-3 in tests conducted with initial 1,2-DCA concentrations of 0.5 mM in serum bottles containing H_2 at an initial concentration of 10% (v/v) (as opposed to the 80% (v/v) employed in the present study) (Yan *et al.*, 2009). All reductive dechlorination reactions known to date for these strains appears to involve dihaloelimination reactions (simultaneous removal of halogen from adjacent carbon atoms and consistent formation of a carbon-carbon double bond).

Ethene was detected in abiotic negative controls but in relatively minute quantities (<0.7 μmol/bottle) indicating that abiotic transformation in the anaerobic culture medium employed here was not substantial (see open symbols in Figure 1). The sum of parent compound (i.e., 1,2-DCA) plus daughter product (i.e., ethene) in replicate serum bottles inoculated with the bacterial strains ranged from 74-107% of the mass determined in abiotic negative controls (average = 89%), indicating reasonable mass balance closure.

1,2-DCA reductive dechlorination was observed in serum bottles with initial 1,2-DCA concentrations as high as 9.81±0.98 and 8.69±0.26 mM for strains IP3-3 and BL-DC-9^T, respectively. At higher initial 1,2-DCA concentrations, small amounts of ethene were also detected, but in amounts that were not statistically different from abiotic negative controls at a confidence level of 95%. The P-values were 0.125 and 0.073 for IP3-$3^T$and BL-DC-9^T at the next highest concentration tested, respectively.

The quantity of ethene observed after eight weeks incubation in the serum bottles increased with increasing 1,2-DCA concentration in the range of 0.5 to approximately 4 mM and then decreased at higher initial 1,2-DCA concentrations. Maximum ethene concentrations were observed in bottles containing initial 1,2-DCA concentrations of 4.03±0.09 and 4.08±0.16 mM

for IP3-3and BL-DC-9T, respectively. The decrease in ethene production as 1,2-DCA concentrations increased indicates that a sufficiently high 1,2-DCA concentrations can inhibit dechlorination activity of both *Dehalogenimonas* spp.

3.2 1,2-DCP Dechlorination as a Function of Solvent Concentration

When provided with 1,2-DCP at initial aqueous-phase concentrations less than 3.19±0.20 mM and 2.14±0.12 mM, dechlorination of 1,2-DCP to a final product of propene was essentially complete in bottles inoculated with IP3-3 and BL-DC-9T, respectively, with <1% of the starting 1,2-DCP remaining at the end of the eight week incubation period (Figure 2). At higher initial 1,2-DCP concentrations, denoted by arrows in Figure 2, 1,2-DCP remained at the end of eight weeks.

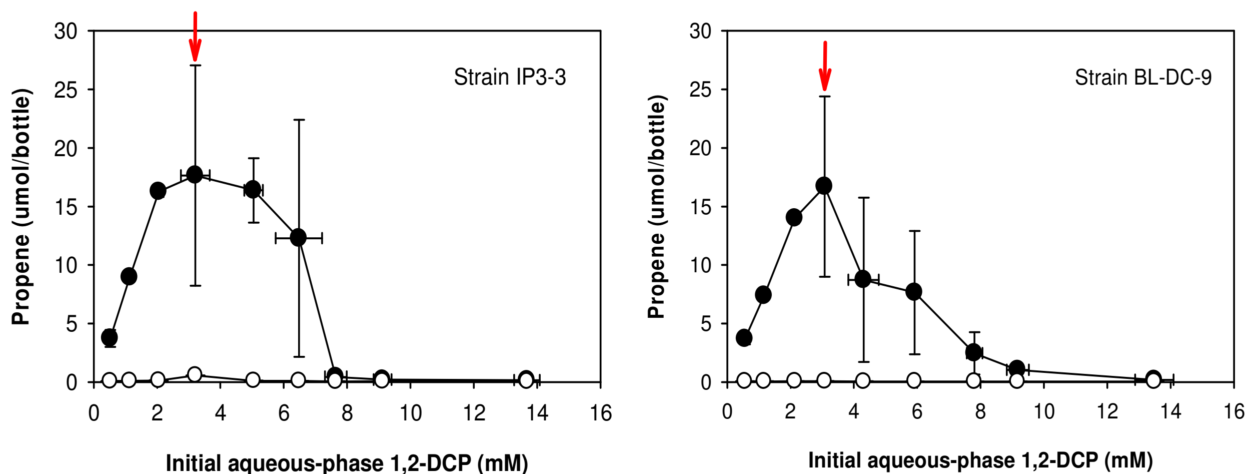

Figure 2. Concentration range study of reductive dechlorination of 1,2-DCP by strains IP3-3 and BL-DC-9T after 8 weeks. Vertical error bars represent one standard deviation in final measurements of triplicate bottles. Horizontal error bars represent standard deviation of initial concentration in triplicate bottles. Filled symbols denote inoculated (biotic) bottles and open symbols denote uninoculated (abiotic) negative controls.

Propene was detected in abiotic negative controls but in relatively minute quantities (<0.13 µmol/bottle, see open symbos in Fig. 2). The sum of parent chlorinated solvent (i.e., 1,2-

DCP) and daughter products (i.e., propene) in replicate bottles inoculated with the bacterial strains ranged from 74-131% of the mass determined in abiotic negative controls (average = 95%), indicating good mass balance closure. This is consistent with the dihaloelimination degradation pathways previously reported for strains BL-DC-9^T and IP3-3 in tests conducted with 0.5 mM 1,2-DCP in serum bottles containing 10% v/v H_2 in the gas headspace (Yan *et al.*, 2009; K.S. Bowman, personal communication).

Trace levels of 1-chloropropane (<0.03 µmol/bottle) were detected in bottles inoculated with strains BL-DC-9^T and IP3-3 and uninoculated abiotic controls, indicating small amounts of abiotic degradation from 1,2-DCP. In mixed culture experiments conducted by Löffler *et al.* (1997), 1-Chloropropane (1-CP) and 2-Chloropropane (2-CP) were detected after 4 weeks of incubation in samples spiked with 1,2-DCP. These dechlorination intermediates were subsequently degraded to propene after 4 months. When samples that had previously degraded 1,2-DCP to propene were spiked with either 1-CP or 2-CP, the monochlorinated propanes were converted to propene.

Similar to what was observed with 1,2-DCA, the quantity of propene formed from 1,2-DCP dechlorination increased at initial 1,2-DCP concentrations ranging from 0.5 to roughly 3 mM and then decreased at higher initial 1,2-DCP concentrations. The highest propene concentration was observed in bottles containing initial 1,2-DCP concentrations of 3.21±0.46 and 3.08±0.05 mM for strain IP3-3 and BL-DC-9^T, respectively. This indicates that beyond a certain threshold, as was observed with 1,2-DCA, 1,2-DCP became inhibitory to dechlorination activity of these strains.

Nevertheless, 1,2-DCP reductive dechlorination was observed in serum bottles with initial 1,2-DCP concentrations as high as 5.05±0.29 and 4.02±0.09 mM for strains IP3-3 and BL-DC-9^T, respectively. At higher initial 1,2-DCP concentrations, small amounts of propene were

also detected, but in amounts that were not statistically different from abiotic negative controls at a confidence level of 95%. The P-values were 0.173 and 0.161 for IP3-$3^T$and BL-DC-9^T at the next highest concentration tested, respectively.

3.3 1,1,2-TCA Dechlorination as a Function of Solvent Concentration

As shown in Figure 3, when strains IP3-3 and BL-DC-9^T were initially supplied with 1,1,2-TCA aqueous-phase concentrations below 2.42±0.22 mM and 1.65±0.03 mM, respectively, dechlorination was complete after 8 weeks of incubation, with trace amounts of 1,1,2-TCA still remaining. At higher concentrations, indicated with the red arrow in Figure 3, 1,1,2-TCA was still present at the end of the incubation period.

Trace levels of 1,2-DCA (< 1.5 µmol/bottle) were present in the bottles inoculated with strains IP3-3 and BL-DC-9^T and uninoculated, negative controls, indicating small amounts of abiotic degradation from 1,1,2-TCA. Vinyl chloride was also present in abiotic negative controls in relatively minute quantities (<1 µmol/bottle). The sum of the parent solvent being tested (i.e. 1,1,2-TCA) and the daughter products (i.e, and vinyl chloride) in replicate bottles inoculated with the bacterial strains ranged from 74-146% of the mass determined in abiotic negative controls (average = 99%), indicating very good mass balance closure. This finding is consistent with the previously reported dihaloelimination degradation pathways for strains BL-DC-9^T and IP3-3 in tests conducted with 0.5 mM 1,1,2-TCA in serum bottles containing 10% v/v H_2 in the gas headspace (Yan *et al.*, 2009; K.S. Bowman, personal communication).

Similar to what was observed with 1,2-DCA and 1,2-DCP, the quantity of vinyl chloride formed from 1,1,2-TCA dechlorination increased at initial 1,1,2-TCA concentrations ranging from 0.5 to roughly 2 mM and then decreased at higher initial 1,1,2-TCA concentrations. Maximum vinyl chloride concentrations were observed in bottles containing initial 1,1,2-TCA concentrations of 1.82±0.18 and 1.65±0.03 mM for strain IP3-3 and BL-DC-9^T, respectively.

This indicates that beyond a certain threshold, as was previously observed with 1,2-DCA and 1,2-DCP, 1,1,2-TCA became inhibitory to dechlorination activity.

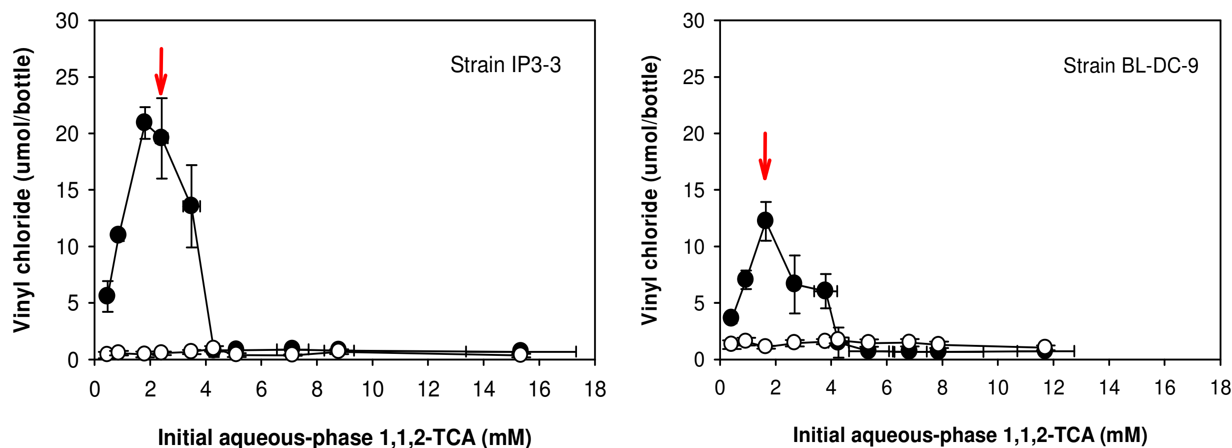

Figure 3. Concentration range study of reductive dechlorination of 1,1,2-TCA by strains IP3-3 and BL-DC-9^T after 8 weeks. Vertical error bars represent one standard deviation in final measurements of triplicate bottles. Horizontal error bars represent standard deviation of initial concentration in triplicate bottles. Filled symbols denote inoculated (biotic) bottles and open symbols denote uninoculated (abiotic) negative controls.

However, 1,1,2-TCA reductive dechlorination was observed in serum bottles with initial 1,1,2-TCA concentrations as high as 3.49±0.31 and 3.80±0.42 mM for strains IP3-3 and BL-DC-9^T, respectively. At higher initial 1,1,2-TCA concentrations, small amounts of vinyl chloride were also detected, but in amounts that were not statistically different from abiotic negative controls at a confidence level of 95%. The P-values were 0.734 and 0.809 for IP3-3^{T}and BL-DC-9^T at the next highest concentration tested, respectively.

Hydrogen (H_2) remained at concentrations >62% (v/v) at the end of the eight-week incubation period for all chlorinated solvent concentrations tested for all three compounds tested for both strains, indicating that it was not stoichiometrically limiting.

3.4 1,2-DCA, 1,2-DCP, and 1,1,2-TCA Track Studies

The primary reason for conducting these experiments was to monitor the dechlorination of these compounds over the course of the 8 week concentration range experiment. Results

showed more than 70% of the initial parent compound is degraded by the 5 week time step measurement. When strains IP3-3 and BL-DC-9^{T} were supplied with initial aqueous phase concentrations of approximately 2 mM of 1,2-DCA, 1,2-DCP, and 1,1,2-TCA in these track studies, dechlorination was completed by the end of the 8 week incubation period for all combinations of contaminants and organisms.

3.5 Discussion

As a basis for comparing the concentrations tested here relative to saturation concentrations, 1,2-DCA solubility in water at 20ºC is 86.1 mM (Horvath *et al.*, 1999). 1,2-DCP solubility in water at 20ºC is 23.9 mM (Horvath *et al.*, 1999). 1,1,2-TCA solubility in water at 20ºC is 32.9 mM (ASTDR, 1989). Also as a basis for comparison, groundwater in the well from which *D. lykanthroporepellens* strain BL-DC-9^{T} was isolated had 1,2-DCA, 1,2-DCP, and 1,1,2-TCA concentrations that averaged 5.5 mM (range: 3.7 to 7.6 mM), 0.6 mM (range: 0.5 to 0.7 mM), and 2.8 mM (range: 1.8 to 4.0 mM), respectively (Bowman *et al.*, 2006; Yan *et al.*, 2009). Results determined here indicate that both *D.* lykanthroporepellens strain BL-DC-9 and *Dehalogenimonas* strain IP3-3 can reductively dehalogenate 1,2-DCA and 1,2-DCP at concentrations even higher than those present in the DNAPL source zone area of the Brooklawn portion of the PPI site. The high end of the range of 1,1,2-TCA concentrations found in this source zone approximately coincides with the highest concentration found in this study to be reductively dechlorinated by both organisms.

Strain IP3-3 was isolated from groundwater contaminated with 1,2-DCA, 1,2-DCP, and 1,1,2-TCA at concentrations of 0.023 mM, 0.022 mM, and 0.010 mM, respectively (unpublished data). Although initially isolated from an environment with much lower chlorinated solvent concentrations than *D. lykanthroporepellens* strain BL-DC-9^{T}, results from the present study

demonstrate that strain IP3-3 can reductively dechlorinate 1,2-DCA, 1,2-DCP, and 1,1,2-TCA at concentrations comparable to BL-DC-9^{T}.

The toxicity of non-chlorinated solvents (e.g., alcohols, alkanes, and aromatic hydrocarbons), has been previously correlated to hydrophobicity as measured by the log of partition coefficients in a standard octanol/water system, log K_{ow} (Sikkema *et al.*, 1995). Compounds with log K_{ow} between 1.5 and 4 are generally toxic to microorganisms (Inoue and Horikoshi, 1991; Sikkema *et al.*, 1994), with maximum toxicity exhibited by compounds with log K_{ow} between 2 and 4 (Kieboom and de Bont, 2000). The adverse effects of 1,2-DCA, 1,2-DCP, and 1,1,2-TCA on reductive dechlorination by the bacterial strains tested here are consistent with these previous observations. For equal molar concentrations, 1,1,2-TCA (log K_{ow} 2.47, Alvarez and Illman, 2006) was more inhibitory than 1,2-DCP (log K_{ow} 2.0, Alvarez and Illman, 2006) which had a larger adverse effect than 1,2-DCA (log K_{ow} 1.48, Alvarez and Illman, 2006).

The ability of *Dehalogenimonas* spp. to reductively dechlorinate high concentrations of halogenated alkanes has important implications for cleanup of contaminated soil and groundwater. Abiotic transformation of these chemicals in the environment is generally quite slow. For example, the environmental half-life of 1,2-DCA from abiotic transformation in water was estimated to be 50 years (Vogel *et al.*, 1987). Unlike chlorinated ethenes (e.g., tetrachloroethene (PCE) and trichloroethene (TCE)), several of the polychlorinated ethanes and propanes, 1,2-DCA in particular, are resistant to transformation by zero-valent iron (Song and Carraway, 2005; Sarathy *et al.*, 2010), limiting physico-chemical remediation approaches for cleanup. The fact that *Dehalogenimonas* spp. are able to perform reductive dechlorination even in the presence of high concentrations of chlorinated compounds suggests that they may provide an important role in bioremediation.

CHAPTER 4 CONCLUSIONS AND FUTURE WORK

4.1 Conclusions and Implications

Both species were shown to dechlorinate 1,2-DCP, 1,2-DCA, and 1,1,2-TCA at initial concentrations at least as high as 8.7 mM, 4.0 mM, and 3.5 mM, respectively. When compared to the concentrations of these contaminants in the area from which these bacteria were isolated from, the organisms were shown to be capable of dehalogenating concentrations at least as high as those present. These results have important implications for the remediation of contaminated soil and groundwater at the PPI Superfund site and many other locations around the world.

4.2 Proposed Future Work

In the course of studying these organisms and their ability to reductively dehalogenate previously untested concentrations of chlorinated alkanes, ideas for future work were abundant. One recommendation for future research is to conduct additional studies to determine how these organisms will perform in a real world bioaugmentation scenario. The conditions provided in the experiments described in this thesis, such as the nutrients, antibiotics, high hydrogen levels, and temperature of 30°C are not generally found in the sites that these organisms might be applied for bioremediation. While it is possible to amend sites with some of these growth-enhancing compounds, control of some parameters (e.g. temperature) could prove cost prohibitive in many situations. Further research into this topic could help engineers and scientists to design better treatment plans and systems to treat these sites cost-effectively using these organisms.

It is recommended that future research should include contaminant mixtures as opposed to single contaminants tested in the present work. While good for gaining insights into the metabolic potential of these organisms, the use of optimal growth conditions and single contaminant experiments may fail to adequately characterize behavior in systems with less ideal

conditions and complex contaminant mixtures. Though it would be inefficient to attempt to replicate the contaminant mixtures and concentrations present at all sites, a general body of work associated with mixtures and these organisms would provide a solid base of information to work from for future site remediation plans.

In work described in this thesis, the *Dehalogenimonas* strains were tested as pure cultures. In practice, these organisms exist in mixed cultures and it may be desirable to supply them as mixed cultures in future bioaugmentation endeavors. This may allow a site to setup a pilot scale study with a small sample of organisms to work with the mixtures present at their site and determine the effects of these different organism mixtures on their particular site dynamics.

Further research into how to grow large volumes of these organisms cost effectively and systematically for injection into a contaminated site would be invaluable to those working to remediate these sites. To replicate the 3% (v/v) inoculations of organisms into the 10 mL of aqueous volume for the tests performed with this work, very large volumes would be needed to treat a site that has similar contamination. To reduce this volume to more manageable levels, further investigation into the effect of organism inoculation level needed to initiate in-situ degradation of various contaminants would like prove worthwhile. Saving a fraction of a percent of injection volume could mean a large cost savings on chemicals, incubators, pumps, etc. needed to grow and inject microbes at the field scale.

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APPENDIX TRACK STUDY RESULTS

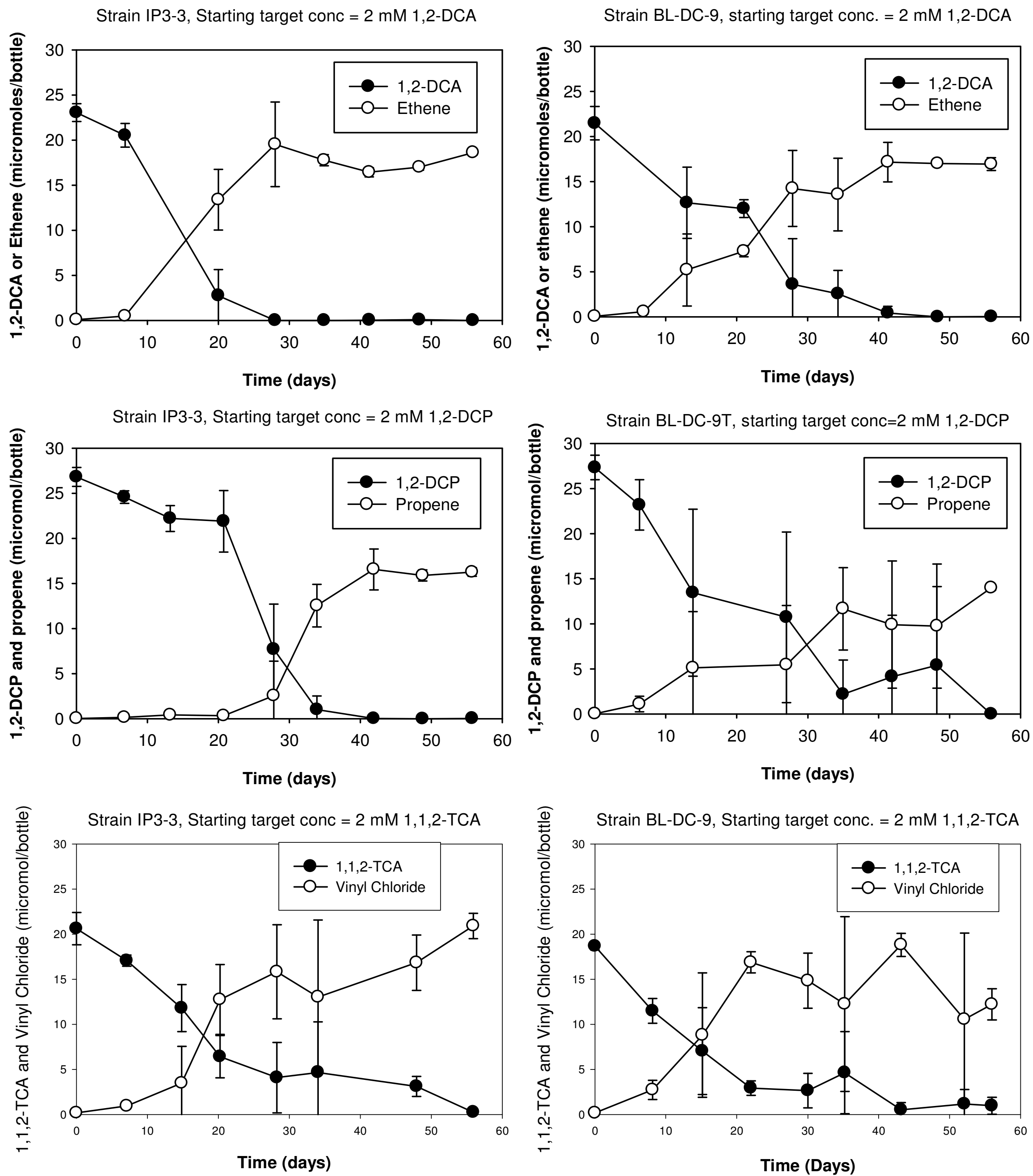

Figure A-1. Results from 1,2-DCA (top row), 1,2-DCP (middle row) , and 1,1,2-TCA (bottom row) track studies using strains IP3-3 and BL-DC-9^{T}.

VITA

Andrew was born in Mobile, Alabama in 1988. He is the son of James and Charlotte Maness. He moved to Baton Rouge, Louisiana, when he was 6 years old and has lived here since that time.

He graduated with honors from Parkview Baptist High School in Baton Rouge, Louisiana, in May, 2006. He then enrolled at LSU as a biological engineering major. In May, 2010, Andrew graduated *Cum Laude* in biological engineering. He then entered the civil engineering master's program the following August. He began research in Dr. Bill Moe's lab and began taking classes to fulfill the requirements for a Master of Science in Civil Engineering.

During his career at LSU, Andrew only missed one home LSU football game. He attended a game at all 12 SEC football stadiums that LSU plays in. At one time, Andrew had a streak of attending 34 straight LSU football games, home and away. He traveled as far west as Seattle, Washington, and as far east as Columbia, South Carolina, to watch his Tigers play. He had tickets on the second and first rows for the 2007 and 2011 BCS National Championship games, respectively. With all that said, Andrew loves LSU football and cannot wait for the fall to come every year.

Also during his career at LSU, Andrew picked up a hobby that turned itself into a business. For Christmas 2006, Andrew asked for a used digital SLR camera. He received one, as well as a simple, inexpensive lens to go along with it. Over time, as he was able to save some money, Andrew had the urge to try to shoot high school sporting events and sell the pictures to the parents to fund more lenses and other photography gear. Starting with the 2009-2010 PBS boys high school basketball season, where Andrew attended high school, he had his first official team to work with. Over the course of the next 3 seasons, Andrew would become the official

team photographer, begin covering almost every event the team had, and would even make gifts for the team booster club to give to coaches, players, and parents who were deserving every year.

As he began to become more comfortable in photographic abilities, Andrew began to branch out into other areas of the photographic industry. In the fall of 2011, Andrew began to offer senior portraits and began to help professional wedding photographers to more accurately understand the industry. Of the PBS graduating class of 2012, Andrew took senior portraits for 8 of the graduating seniors. Also, in the spring of 2012, Andrew started accepting bookings for wedding photography. As of this document, he has booked 4 weddings in the next 8 months and has had inquiries for other dates into 2013.